

From capsules to nutlets—phylogenetic relationships in the Boraginales

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Abstract

Multiple family-level subdivisions of Boraginales have been proposed in the past. The relationships of several constituent genera have been enigmatic, including *Codon* (Codonaceae), *Hoplostigma* (Hoplostigmataceae), *Pholisma* (Lennoaceae), *Vahlia* (Vahliaceae), and *Wellstedia* (Wellstediaceae), all of which are included in the present study. We present a molecular analysis with four chloroplast loci, including 89 ingroup taxa and a broad outgroup sampling in the asterids. The genus *Vahlia* is excluded from Boraginales and appears to represent an early branching lineage of Lamiales. The study provides a well supported topology for the relationships within Boraginales, including all of the genera with previously unclear relationships. Within Boraginales, two major clades are recognized, with “herbaceous” Boraginales I resolved as [Codonaceae,[Wellstediaceae,[Boraginaceae]]] and “woody” Boraginales II resolved as [Hydrophyllaceae I,[Hydrophyllaceae II,[Heliotropiaceae,[Cordiaceae, [Ehretiaceae,Lennoaceae]]]]. A close relationship between Ehretiaceae and Lennoaceae is well supported, but the exact placement of Lennoaceae remains unresolved. The Cordiaceae lineage includes the monotypic genus *Coldenia* and the aberrant western and central African genus *Hoplostigma*. Woody Boraginales II are retrieved in two highly supported clades. Hydrophyllaceae are retrieved in two separate clades, but with poor support. There appear to be clear morphological progressions in vegetative, floral, and fruit morphology in both major Boraginales lineages. Thus capsular fruits are found in the first branching lineages of both clades, whereas reduced seed numbers in indehiscent fruits predominate in the more derived phylogenetic positions. Based on these results, we advocate the recognition of eight morphologically well defined clades in the order, namely Boraginaceae s.str., Codonaceae, Cordiaceae (incl. *Coldenia* and Hoplostigmataceae), Ehretiaceae (incl. Lennoaceae), Heliotropiaceae, Hydrophyllaceae I and Hydrophyllaceae II, and Wellstediaceae.

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Introduction

Boraginales (= Boraginaceae s.l. of various authors) are a morphologically diverse group of angiosperms comprising approximately 2450 species (Mabberley, 2008). They are cosmopolitan in distribution, with the

majority of species occurring in seasonally arid habitats across temperate and tropical regions. Like many representatives of the asterids, the Boraginales have mostly tetracyclic, sympetalous, and pentamerous flowers, predominantly with radial symmetry and frequently arranged in scorpioid inflorescences (Buys and Hilger, 2003). Fruit morphology is particularly diverse across the Boraginales, comprising capsules, drupes, nutlets, and schizocarps. Complex fruit architecture

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results from the development of various secondary septa subdividing the bicarpellate gynoecium (Hilger 1985, 1992; Gottschling, 2004).

Historically, de Candolle (1845, 1846), Bentham and Hooker (1876), and Gürke (1893) treated Boraginaceae in a broad taxonomic sense, recognizing four subfamilies: Boraginoideae, Cordioideae, Ehretioideae, and Heliotropioideae. This subdivision was followed in the majority of subsequent studies, including those of Ivan M. Johnston in his 31 “Studies in the Boraginaceae” (indexed in Hilger and Zippel, 2001; Miller et al., 2005). Gürke (1893) emphasized the close relationship of Hydrophyllaceae to Boraginaceae. Takhtajan (1987) recognized the order Boraginales with seven families, including Boraginaceae, Cordiaceae, Ehretiaceae, Hoplostigmataceae, Hydrophyllaceae, Lennoaceae, and Wellstediaceae. He later revised the Boraginales (Takhtajan, 1997) to include Boraginaceae with five subfamilies (Boraginoideae, Cordioideae, Ehretioideae, Heliotropioideae, and Wellstedioideae), Hoplostigmataceae, Hydrophyllaceae, Lennoaceae, and Tetrachondraceae.

More recent molecular studies have shown that Tetrachondraceae (Wagstaff et al., 2000), as well as individual genera of Hydrophyllaceae and Boraginaceae s.l. such as *Hydrolea* L. and *Pteleocarpa* Oliv., are best removed from Boraginales. *Hydrolea* is not closely related to any other member of the family and is best placed in Solanales, allied with the Sphenocleaceae and Montiniaceae (Cosner et al., 1994; Soltis et al., 2000). *Pteleocarpa* is now considered a member of the Gentianales (Riedl, 1997; Mabberley, 2008; Brummitt, 2011; Refulio-Rodriguez and Olmstead, unpublished sequence data from NCBI GenBank). The remaining Boraginaceae s.l. are monophyletic only when the Hydrophyllaceae and parasitic Lennoaceae are included, although each of the formerly recognized subfamilies is monophyletic (Ferguson, 1999; Gottschling et al., 2001). The recognition of a broadly defined Boraginales, comprising the four subfamilies of the Boraginaceae s.l. elevated to family rank (i.e. Boraginaceae s.str., Cordiaceae, Ehretiaceae, and Heliotropiaceae) as well as the Hydrophyllaceae (excluding *Hydrolea*) and Lennoaceae, has also been suggested by various authors (Gottschling et al., 2001, 2005; Luebert and Wen, 2008; Mansion et al., 2009).

Gürke (1893) considered Cordiaceae, Ehretiaceae, and Heliotropiaceae as first lineages of the Boraginales, but they have since been shown to constitute a derived monophyletic group together with the Lennoaceae (Primarily Woody Boraginales: Gottschling et al. 2004). The more derived status of the Primarily Woody Boraginales is also underscored by anatomical data, as these groups share a multilayered, hard endocarp (otherwise rare in asterids) and specialized

transfer cells in the seed coat as apomorphic characters (Diane et al., 2002b).

Molecular sequence comparison has contributed to an increasingly detailed view of the relationships within Boraginaceae s.str. (e.g. Langström and Chase, 2002; Weigend et al., 2009, 2010). Moreover, *Codon* L. has traditionally been regarded as the only African member of Hydrophyllaceae, but it is aberrant for the family (and the order) in its spiny habit, numerous sepals, petals, and stamens, and shares only the apical style and the many-seeded loculicidal capsule with Hydrophyllaceae (Weigend and Hilger, 2010). Its two species occur in Namibia and neighbouring South Africa, and the taxon is now regarded as the family Codonaceae, closely allied to Boraginaceae s.str. (Weigend and Hilger, 2010). Molecular data also support additional generic realignments within the Boraginales, such as the inclusion of *Auxemma* Miers, *Patagonula* L., and *Saccellium* Humb. & Bonpl. in *Cordia* L. (Gottschling et al., 2005; Gottschling and Miller, 2006); the segregation of *Varronia* L. from *Cordia* (Miller and Gottschling, 2007); the inclusion of *Carmona* Cav., *Cortesia* Cav., and *Rotula* Lour. in *Ehretia* L. (Gottschling and Hilger, 2001, 2004); the resurrection of *Hilsenbergia* Tausch ex Meisn. (Miller, 2003); and the segregation of *Euploca* Nutt. and *Myriopus* Small from *Heliotropium* L. and *Tournefortia* L., respectively (Hilger and Diane, 2003; Luebert et al., 2011b).

Beyond these new alignments, the systematic position of a number of enigmatic taxa has remained unclear. *Wellstedia* Balf.f. comprises six species (Thulin and Johansson, 1996; Thulin, 1998) that occur from Ethiopia to Somalia and Socotra, with a disjunct species in western South Africa. The genus has been recognized as a monogeneric family Wellstediaceae allied with Boraginales, but differs in its two-locular ovary that develops into a one- to two-seeded, septifragal then ventricidal capsule. It is also unusual in having tetramerous flowers. *Hoplostigma* Pierre comprises two species occurring in West and Central Africa, and its relationships have been unclear (Nowicke and Miller, 1989). The genus has been placed in Bixales (Hutchinson, 1959), Ebenales (Wagenitz, 1964), Violales (Cronquist, 1981), and Boraginales (Takhtajan, 1987). It shares the basic perianth morphology of Boraginales, but differs in having 20–35 stamens that are attached in three irregular series to the short corolla tube, and in having a unilocular ovary with four ovules. Nowicke and Miller (1989) found the pollen of *Hoplostigma* to be similar to that of *Bourreria* P.Br. and *Ehretia* of the Ehretiaceae. Some molecular studies have also suggested a sister group relationship between Vahliaaceae and Boraginales (Lundberg, 2001; Bremer et al., 2002). *Vahlia* Thunb. is morphologically anomalous for Boraginales in having opposite leaves, free petals,

and an inferior ovary that is unilocular with apical placentae. *Vahlia* includes eight species of herbs and subshrubs from tropical and southern Africa and India (Bridson, 1974), and was placed in Saxifragales by Cronquist (1981) and Takhtajan (1987).

APG III (2009) classifies Boraginaceae s.l. (Boraginales sensu Gottschling et al., 2001) as “unplaced” in the lamiid clade, and in a close but unresolved relationship to Gentianales and more distantly to Solanales and Lamiales. In contrast to our opinion, APG III (2009) defines Boraginaceae in a very broad sense, including taxa with capsular fruits as well as with fruits in four-one-seeded nutlets and with apical as well as with gynobasic styles, and also includes *Hoplostigma*, although without published evidence. The present study aims at improving the phylogenetic understanding of Boraginales, with an emphasis on crucial taxa, for which molecular data have been either completely absent, or incomplete and based on a small sample. We aim at a broad sampling using four plastid loci in order to elucidate the relationships of Boraginales. This is the first study with a comprehensive sampling of all of the major lineages in Boraginales. Previous works have generated trees with topologies similar to that presented here, but consistently lacked the sampling and/or the resolution to answer the questions addressed here (Ferguson, 1999; Gottschling and Hilger, 2001; Diane et al., 2002a, Luebert et al., 2011a; Nazaire and Hufford, 2012). One notable exception is a highly resolved phylogeny of Boraginaceae s.str., which largely resolved the internal relationships of that family (Weigend et al., 2013). The present paper can be seen as the logical extension of that paper on the wider relationships in Boraginales.

Materials and methods

Plant material

In total, 134 accessions were investigated (Appendix S1). Within Boraginales, sampling is broad and includes representatives of all relevant groups, with at least one (*Hoplostigma*; *Pholisma* Nutt. ex Hook.) or several species (e.g. both species of *Codon*: Weigend and Hilger, 2010; three of the six species of *Wellstedia*: Thulin and Johansson, 1996; Thulin, 1998). Within major groups, a broad sampling of genera is included, with an emphasis on the putatively first diverging taxa recognized in previous studies. Three species of *Vahlia* were included to test its possible sister group relationship to Boraginales. Outgroup selection was based on previous studies, including representatives of all orders from the lamiid clade (APG III, 2009). Some representatives of the campanulid clade were also included as sister to the lamiid clade, and the final trees were

rooted using members of the Cornales and Ericales, which are successive sister groups to lamiids and campanulids within the asterids (APG III, 2009). Plant material was obtained from silica-dried samples collected in the field and/or from cultivation, and additional material was obtained from herbarium specimens. GenBank accessions were also downloaded to complete the data set, especially in the outgroups.

Phylogenetic analyses

Phylogenetic analyses were conducted using a data matrix with four plastid loci (*ndhF*, *rbcL*, *rps16*, and *trnL-trnF*). We included only accessions with all four loci sequenced (i.e. no missing data), with the exception of *Hoplostigma* (*rps16* missing) and *Pholisma* (*ndhF* missing) because of their systematic importance. The data set was partitioned into four parts, and the nucleotide sequences (and alternatively amino acid sequences of *rbcL* and *ndhF*) were aligned using MAFFT v.6.624b (Katoh et al., 2005; freely available at <http://mafft.cbrc.jp/alignment/software/>) and concatenated afterwards. The consideration of highly divergent sequences such as *rps16* and *trnL-trnF* over a broad taxonomic range should be treated with caution, and we explored the possible negative effects for our phylogenetic reconstructions by excluding phylogenetically ambiguous positions by filtering the corresponding matrices using GBLOCKS (Castresana, 2000) with various settings. The final data matrices are available on request.

Maximum parsimony (MP) analyses were conducted in PAUP* 4.0b10 (Swofford, 2003), using the parsimony ratchet algorithm (Nixon, 1999) implemented in PRAP 2.0b3 (Müller, 2004) with 200 iterations. Trees were generated by performing heuristic searches with tree bisection–reconnection and starting trees obtained via random stepwise taxon addition sequence, with 1000 replicates. Maximally parsimonious trees (MPTs) were summarized as a strict consensus tree. Maximum parsimony non-parametric bootstrap support (PBS) was estimated based on 1000 replicates using the same search strategy as in the tree searches (to save calculation time, the upper limit for the number of equally parsimonious trees was specified to 100). Gaps were treated as missing data in all analyses. Decay values (Bremer, 1988) were also calculated for the MP strict consensus tree using PRAP 2.0b3.

Bayesian (BA) and maximum likelihood (ML) analyses were carried out using the resources of the SGI system (Zuse Institute Berlin, ZIB), one half of the North German Supercomputing Alliance (HLRN). For the ML calculations, the MPI version of RAxML v.7.2.6 (Stamatakis, 2006; freely available at <http://sco.h-its.org/exelixis/software.html>) was applied using the GTR+ Γ substitution model (or the cpREV substitution

model in case of amino acids: Adachi et al., 2000). To determine best fitted ML-trees, we executed 10-tree searches from distinct random stepwise addition sequence MP starting trees and 1000 non-parametric bootstrap replicates. Bayesian analyses was performed using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003; freely available at <http://mrbayes.csit.fsu.edu/download.php>) for comparability under the same substitution models as in the ML analyses and the random addition-sequence method with 10 replicates. We ran two independent analyses of four chains (one cold and three heated) with 15 000 000 cycles (1 500 000 cycles for amino acid alignments due to time out), sampled every 1000th cycle, with an appropriate burn-in (10%) as inferred from the evaluation of the trace files using Tracer v.1.5 (<http://tree.bio.ed.ac.uk/software/tracer/>). Statistical support values (ML bootstrap support, LBS; Bayesian posterior probabilities, BPP) were drawn on the resulting, best scoring ML tree.

Results

Our nucleotide alignment consisted of 6381 aligned sites (*ndhF*: 2139, *rbcL*: 1359, *rps16*: 1446, *trnL-trnF*: 1437) with 4112 distinct alignment patterns (ML) and 3443 variable sites, of which 2498 were parsimony-informative. Maximally parsimonious trees were 14 443 steps in length, with a consistency index of 0.40 and a retention index of 0.72. Tree topologies are largely congruent, independently of which data processing strategy was applied (Bayesian, ML, or MP algorithm, nucleotide vs amino acid sequences, complete or GBlocks processed alignments), with only one exception of contradictory and at least moderately supported relationships regarding the Hydrophyllaceae (see below). Many nodes exhibit high if not maximal statistical support values (Figs 1 and 2).

Figure 1 depicts the strict consensus tree of the MP analysis, while Fig. 2 shows the best scoring ML tree derived from the nucleotide alignment with statistical support values as an example for the various other analyses. Members of the Cornales and the Ericales are always successive sister groups to the euasterids (100 PBS, 100 LBS, 1.00 BPP), which in turn segregate into the lamiid (euasterid I) clade (100 PBS, 100 LBS, 1.00 BPP) and the campanulid (euasterid II) clade (88 PBS, 81 LBS, 1.00 BPP). Within the lamiid clade, the Boraginales are retrieved as monophyletic in all analyses (100 PBS, 100 LBS, 1.00 BPP), but their closest relatives are not determined unequivocally. In analyses using amino acid sequences, the Boraginales are the sister group of a clade comprising the Solanales and Gentianales (55 LBS, 0.99 BPP; not shown). *Vahlia* is associated

with Lamiales in our analyses (50 PBS, 82 LBS, 0.99 BPP).

The internal relationships of Boraginales show a basic sister group relationship between (i) Boraginales I with two of the monogeneric groups and Boraginaceae s.str. (= subfamily Boraginoideae): [*Codon*, [*Wellstedtia*, Boraginaceae s.str.]] (100 PBS, 100 LBS, 1.00 BPP) and (ii) Boraginales II with paraphyletic (or polyphyletic) Hydrophyllaceae and all other subgroups of Boraginales (91 PBS, 100 LBS, 1.00 BPP). Boraginales I are well resolved with *Codon* and *Wellstedtia* each monophyletic and successive sister groups to the Boraginaceae s.str. (all nodes 100 PBS, 100 LBS, 1.00 BPP). Within Boraginaceae s.str., four lineages are retrieved, with Echiochileae (99 PBS, 100 LBS, 1.00 BPP) sister group to the rest of the family (97 PBS, 95 LBS, 1.00 BPP) and three strongly supported subclades corresponding to Boragineae, Cynoglosseae, and Lithospermeae (all nodes 100 PBS, 100 LBS, 1.00 BPP).

Boraginales II are not fully resolved. Hydrophyllaceae represent first diverging lineages in all analyses and fall into two clades, namely the herbaceous Hydrophyllaceae I (*Eucrypta* Nutt., *Hydrophyllum* L., *Nemophila* Nutt., *Phacelia* Juss., *Pholistoma* Lilja: 100 PBS, 100 LBS, 1.00 BPP in all analyses) and the mostly woody Hydrophyllaceae II (*Nama* L., *Wigandia* Kunth: 100 PBS, 100 LBS, 1.00 BPP in all analyses). Within the remaining Primarily Woody Boraginales, three mostly well supported lineages are retrieved, namely the Cordiaceae (including *Coldenia* L. and *Hoplostigma*: 76 PBS, 96 LBS, 1.00 BPP), the Ehretiaceae (including the Lennoaceae: 66 PBS, 96 LBS, 1.00 BPP), and the Heliotropiaceae (100 PBS, 100 LBS, 1.00 BPP). However, three contradictory and at least moderately supported relationships are worth noting. In nucleotide-based molecular trees, Hydrophyllaceae II are retrieved as sister to Primarily Woody Boraginales (i.e. Cordiaceae, Ehretiaceae incl. Lennoaceae, Heliotropiaceae; 63 LBS, 0.99 BPP), whereas they are the sister group of Cordiaceae and Ehretiaceae (incl. Lennoaceae) in amino acid-based molecular trees (75 LBS, 0.99 BPP; not shown). Maximum parsimony analyses yielded a topology with Hydrophyllaceae I closely related to Cordiaceae and Ehretiaceae but with Hydrophyllaceae II closely related to Heliotropiaceae. None of the analyses retrieves Hydrophyllaceae as monophyletic.

Cordiaceae and Ehretiaceae are retrieved as sister taxa in all analyses with confidence (62 PBS, 88 LBS, 1.00 BPP). Within Cordiaceae, *Coldenia* and *Hoplostigma* are either successive sister taxa of *Cordia* s.l. or together its weakly supported sister group (59 PBS, 60 LBS, 0.90 BPP). *Cordia* s.l. is retrieved (99 PBS, >90 LBS, 1.00 BPP) with *Varronia* (100 PBS, 100 LBS, 1.00 BPP) firmly nested in the crown-clade.

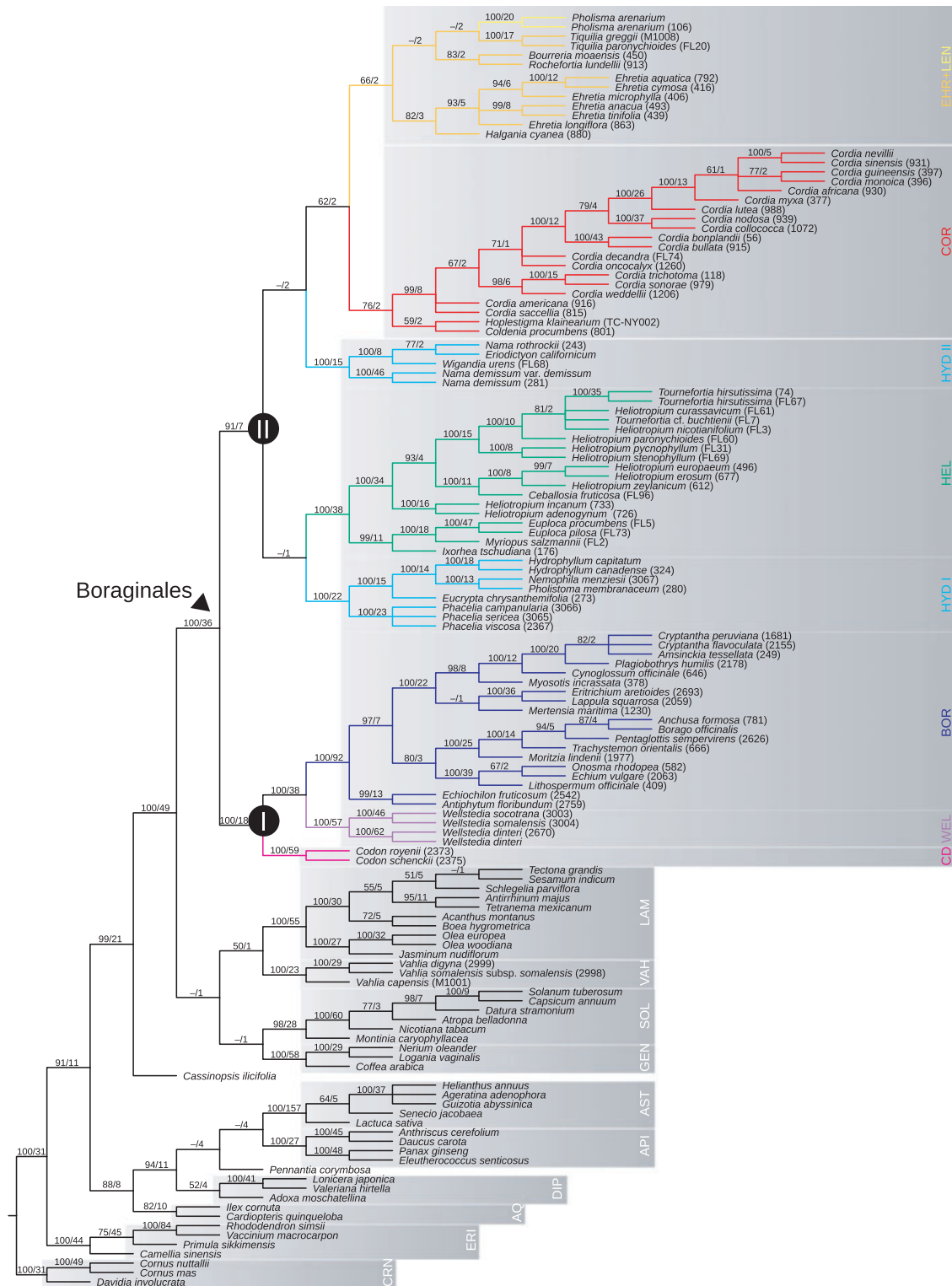


Fig. 1. Strict consensus tree obtained from the maximum parsimony (MP) analysis of Boraginales. Numbers above branches are MP bootstrap values (≥ 50) and decay indices. Major clades are indicated. I, Boraginales I; II, Boraginales II; AST, Asterales; API, Apiales; AQ, Aquifoliales; BOR, Boraginaceae s.str.; CD, Codonaceae; COR, Cordiaceae; CRN, Cornales; DIP, Dipsacales; EHR, Ehretiaceae; ERI, Ericales; GEN, Gentianales; HEL, Heliotropiaceae; HYD I and HYD II, Hydrophyllaceae I and II; LAM, Lamiales; LEN, Lennoaceae; SOL, Solanales; VAH, Vahliaaceae; WEL, Wellstediaceae.

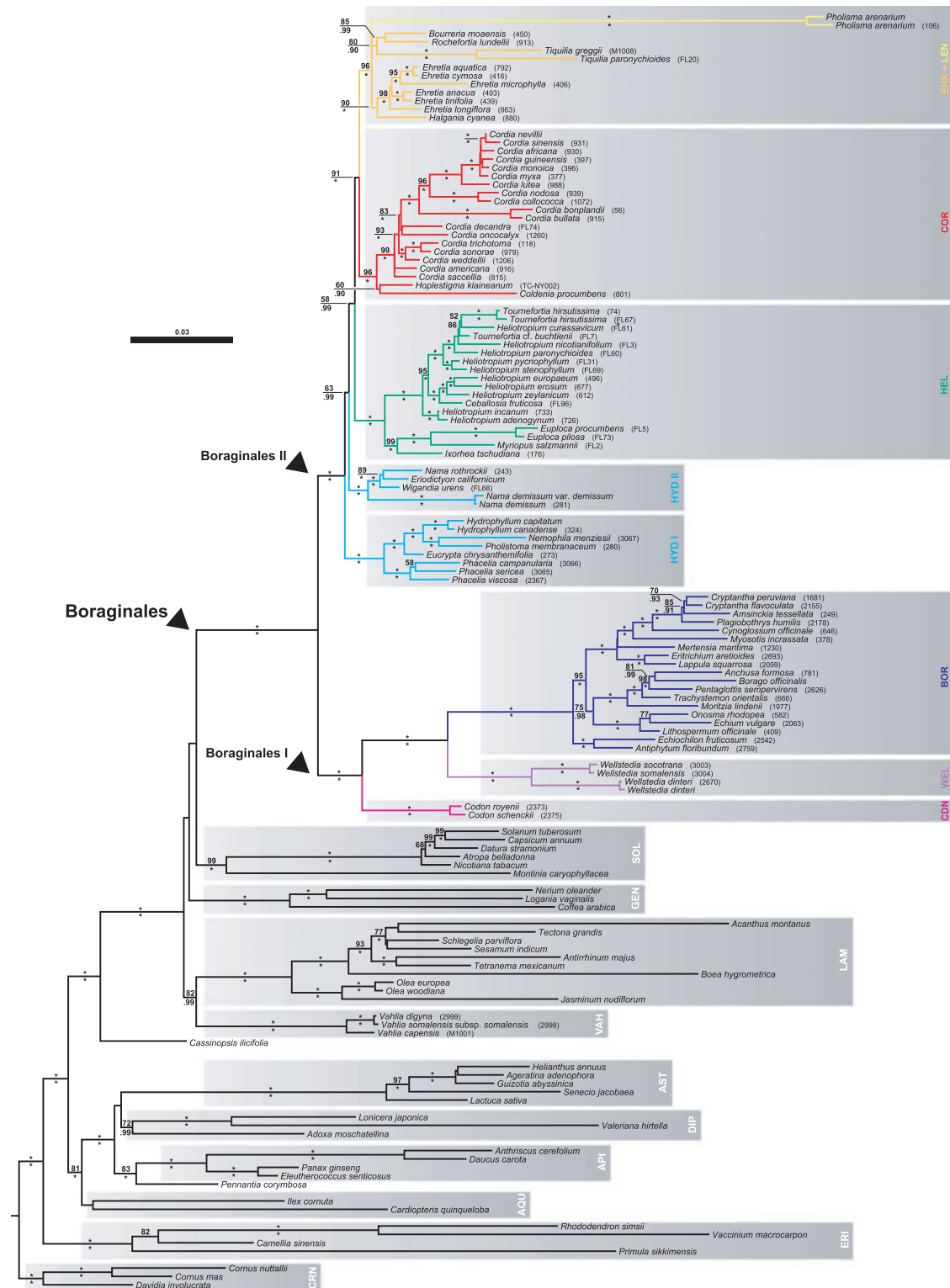


Fig. 2. Maximum likelihood (ML) tree obtained from the analysis in RAxML. Numbers above branches indicate ML bootstrap values (≥ 50) and Bayesian posterior probabilities (≥ 0.90). Asterisks indicate maximal statistical support. Major clades are indicated (API, Apiales; AQU, Aquifoliales; AST, Asterales; BOR, Boraginaceae s.str.; CDN, Codonaceae; COR, Cordiaceae; CRN, Cornales; DIP, Dipsacales; EHR, Ehretiaceae; ERI, Ericales; GEN, Gentianales; HEL, Heliotropiaceae; HYD I and HYD II, Hydrophyllaceae I and II; LAM, Lamiales; LEN, Lennoaceae; SOL, Solanales; VAH, Vahliaceae; WEL, Wellstediaceae).

Within Heliotropiaceae, [*Ixorhea*,*Euploca*,*Myriopus*] are monophyletic (99 PBS, 99 LBS, 1.00 BPP) and the sister group of *Heliotropium* s.l. (including *Tournefortia*: 100 PBS, 100 LBS, 1.00 BPP).

Discussion

Methodological improvement of molecular phylogenetics in the Boraginales

The phylogeny of Boraginales has been controversial since the early days of molecular systematics in the early 1990s. Neither the precise phylogenetic position in the asterids nor the internal relationships have been satisfactorily resolved to date. This study is the first to combine comprehensive taxon sampling evenly covering all major lineages of the Boraginales with an extensive outgroup sampling.

Nazaire and Hufford (2012) recently published the largest analysis so far of the relationships of Boraginales using a “supermatrix”. It is based on a data set with large amounts of empty cells and no single locus sampled comprehensively for all taxa. The original data were taken largely from a range of papers including Ferguson (1999), Gottschling and Hilger (2001), Langström and Chase (2002), Gottschling et al. (2001, 2005), Diane et al. (2003), Hilger et al. (2004), Selvi et al. (2006), Luebert and Wen (2008), Thomas et al. (2008), Mansion et al. (2009), Weigend et al. (2009, 2010), Luebert et al. (2011a), and matrix structure thus closely reflects different taxon subsets with different markers. Simmons (2011) demonstrated that ML analyses may report more highly resolved, but incorrect topologies with greater support from data sets with significant missing data. This is an evident pitfall for the analysis of “supermatrices”, which tend to have a lot of missing data. In the Nazaire and Hufford (2012) analysis, the ML and Bayesian analyses appear to yield well resolved results, but the parsimony analysis provides little—if any—support for many nodes. In contrast, the study presented here is based on a matrix consisting of four plastid loci scored for nearly every terminal taxon. Apart from a few exceptions discussed below, our molecular results are remarkably stable when comparing the results of MP, ML, and Bayesian analyses. We believe that the difference in resolution, topology, and statistical support found between the Nazaire and Hufford (2012) paper and the data here largely go back to these profound differences in raw data structure. In particular, the internal resolution of Boraginaceae s.str. has been clarified in much detail in Weigend et al. (2013), and a comparison of this paper with Nazaire and Hufford (2012) clearly shows the gross differences in resolution and support between the “comprehensive matrix” and

“supermatrix” approach. The present paper demonstrates a similar phenomenon for the remainder of Boraginales.

Relationships of Boraginales

In their current circumscription, Boraginales are doubtlessly monophyletic, and there is firm evidence for its inclusion into the lamiid clade (together with Gentianales, Lamiales, and Solanales). However, our molecular data set (four chloroplast loci) provides only tentative statistical support for a sister group relationship to Gentianales and Solanales (i.e. alignments using amino acids; not shown). Conversely, the weakly supported association of *Vahlia* with Boraginales found by Bremer et al. (2002) is not confirmed. The removal of *Vahlia* from Boraginales and an association with Lamiales agrees with the tentative findings of Albach et al. (2001).

Internal phylogeny of the Boraginales

Within Boraginales, the present study largely confirms and expands the findings on the phylogeny of Boraginales first presented by Ferguson (1999) and Gottschling et al. (2001), with a sister group relationship between the clades here called Boraginales I (Boraginaceae s.str. and Codonaceae, with Wellstediacae added in this analysis); and Boraginales II (Cordiaceae, Ehretiaceae, Heliotropiaceae, two clades of Hydrophyllaceae, Lennoaceae). A similar topology, for only a subset of the taxa sampled here, has been retrieved by Moore and Jansen (2006) and Luebert and Wen (2008). Detailed relationships *within* Boraginaceae s.str. at tribal and generic level have been developed by Hilger et al. (2004: Boragineae), Langström and Chase (2002: Echiochileae), Thomas et al. (2008: Lithospermeae), and Weigend et al. (2009, 2010: Lithospermeae, “Trigonotideae”), and a comprehensive picture is provided by Weigend et al. (2013).

Even after removal of *Codon* and *Hydrolea*, the Hydrophyllaceae are retrieved not as monophyletic, but rather as two clades: primarily herbaceous Hydrophyllaceae I (here and clade I of Ferguson, 1999) and a primarily woody group, Hydrophyllaceae II (here and clade II of Ferguson, 1999). The relationships of these two clades remain unclear, though both groups separately are strongly supported as monophyletic. Chloroplast genes suggest a para- or polyphyletic arrangement of the two groups in the present study as well as previous reports from Ferguson (1999), Moore and Jansen (2006), and Luebert and Wen (2008). A monophyly for Hydrophyllaceae has to be seen with caution, as it is supported by ITS1 sequence data only (Gottschling et al., 2001). Monophyly found in Naz-

aire and Hufford (2012) may indicate once more the problematic application of “supermatrix” approaches for phylogenetic reconstruction.

Heliotropiaceae are monophyletic, and relationships among major clades suggested in previous studies are confirmed (Diane et al., 2002a; Hilger and Diane, 2003; Luebert et al., 2011a,b). The genera *Euploca* and *Myriopus* are sister to each other, and *Heliotropium* is paraphyletic with respect to *Tournefortia* sect. *Tournefortia*. The branching order of major clades within *Heliotropium* [*H.* sect. *Heliothammus*, [Old World *Heliotropium*, [*H.* sect. *Cochranea*, [*Tournefortia*]]]] is also confirmed in this study. Within Heliotropiaceae, the genus *Ixorhea* Fenzl is resolved as sister group of [*Myriopus*, *Euploca*], forming a well supported sister group of *Heliotropium*, as previously shown by Luebert et al. (2011a) and Nazaire and Hufford (2012).

The common ancestry of Cordiaceae, Ehretiaceae, and Lennoaceae has been shown previously (Gottschling et al., 2001) and is also supported by the expanded data set presented here. Lennoaceae are clearly retrieved with Ehretiaceae, as also demonstrated in an analysis covering alternative loci and a much broader taxon sample (M. Gottschling, F. Luebert, H.H. Hilger and J.S. Miller, unpublished). The Lennoaceae are here accepted as part of the Ehretiaceae clade without resolution of whether it is sister to, or nested within, the clade. It had been proposed that *Hoplostigma* has affinities to the woody borages based on morphology (Takhtajan, 1987; Nowicke and Miller, 1989), and its relationship to Cordiaceae is confirmed here for the first time using molecular data. The association of *Coldenia* with Cordiaceae has been reported previously (Gottschling et al., 2005; Moore and Jansen, 2006), and it is confirmed in the present study. While the association of both *Coldenia* and *Hoplostigma* with Cordiaceae is strongly supported, the question of whether they together form the sister group to Cordiaceae or whether the two are progressively sister to *Cordia* is not resolved.

From capsules to nutlets: fruit evolution

It has traditionally been assumed that morphological evolution in the Boraginales has proceeded from the ancestral state of a bicarpellate, superior ovary with a terminal style to a more derived fruit of four nutlets on a persistent gynobase. Following this concept, tropical trees, as present in Cordiaceae and Ehretiaceae, represented the “primitive” condition for Gürke (1893). Our data argue that groups with capsular fruits (i.e. *Codon*, “Hydrophyllaceae”, *Wellstedia*) are not only an integral part of Boraginales, but represent the first branching lineages of both Boraginales

I and Boraginales II. The most parsimonious explanation is that ovule reduction and mericarp/mericarpid formation arose independently in Boraginales I and Boraginales II. This is underscored by the fact that the formation of nutlets is morphologically different between Boraginales I and Boraginales II (Hilger, 1985, 1992; Gottschling, 2004). The interpretation of several independent origins of nutlets in the Boraginales is clearly supported by the phylogenetic trees (see below).

In Boraginales I, Codonaceae are the sister group to [Wellstediaceae, Boraginaceae s.str.], and they have many-seeded, apically dehiscent capsules originating from a superior ovary with an apical style. Wellstediaceae also have a capsular fruit derived from an ovoid ovary with an apical style. Here the number of ovules is, however, reduced to two to four. During fruit development, the lateral parts of the ovary expand, leading to a laterally flattened, heart-shaped capsule with a central depression at the point of style insertion. Both Codonaceae and Wellstediaceae release their seeds from dehiscent capsules. In Boraginaceae s.str., there is an early ontogenetic subdivision of the ovary leading to four independent nutlets surrounding a gynobasic style and later detaching from the gynobase (Hilger, 1985).

In Boraginales II, a more or less parallel scenario can be deduced. The two-first branching lineages of Boraginales II, “Hydrophyllaceae”, have capsular fruit with four or more seeds, probably the plesiomorphic condition and also typical of the bulk of the orders with which Boraginales has been associated (especially Solanales, but also Gentianales and Lamiales). In Primarily Woody Boraginales, four-seeded fruits falling into two or four dry nutlets are found in most Heliotropiaceae (Hilger, 1992) and some Ehretiaceae (*Tiquilia*: Richardson, 1979) and Cordiaceae (*Coldenia*), but phylogenetic homology to the nutlets of Boraginaceae s.str. is unlikely in the light of the present molecular phylogenies. In Cordiaceae, Ehretiaceae, and Heliotropiaceae, drupaceous fruits occur in several groups, with the endocarp either undivided (*Cordia*, *Ehretia* p.p., *Hoplostigma*) or falling into two-two-seeded or four-one-seeded pyrenes (*Bourreria*, *Ehretia* p.p., *Myriopus*, *Tournefortia* sect. *Tournefortia*). Intermediate forms with initially fleshy and later dry mesocarp (especially in some hydrochorous species of *Heliotropium*, but also in *Bourreria* p.p.) and a more or less distinctive endocarp are found in *Coldenia*, *Heliotropium*, and *Tiquilia*. Secondary multiplication and subdivision of the carpels in Lennoaceae leads to a complex ovary and fruit morphology (Yatskievych and Mason, 1986), which is clearly a derived condition. Overall, the entire Cordiaceae–Ehretiaceae clade has a continuous range of drupes to nutlets. Thus both Boraginales I and Boraginales II have a predominance of variously modified

four-seeded schizocarps, but their sister groups have conventional capsules.

Vegetative morphology

Vegetative morphology is highly variable across the order. In Boraginales I, there appears to be a clear progression: the early diverging groups Codonaceae, Wellstediaceae, and Boraginaceae-Echiochileae are mostly suffruticose/fruticose desert plants. The other three tribes of Boraginaceae include a wide range of growth forms (ephemeral to arborescent), with perennial deserticolous taxa largely restricted to Lithospermeae and Cryptanthaeae/Eritrichieae, and a dominance of annual and perennial herbs in Boragineae and Cynoglosseae.

In Boraginales II, the situation is more complex. Hydrophyllaceae I are primarily annual and perennial herbs, whereas Hydrophyllaceae II are primarily shrubby. Many Heliotropiaceae (*Heliotropium* sect. *Heliothamnus* I.M.Johnst., *Myriopus*, *Tournefortia* sect. *Tournefortia*), all Ehretiaceae, and all Cordiaceae except *Coldenia*, are trees or shrubs. Perennial and annual herbs occur in Heliotropiaceae (many *Euploca* and *Heliotropium*), but rarely in Cordiaceae (*Coldenia*), and Ehretiaceae (*Tiquilia* p.p.). These differences in growth habit correlate to habitat, with small-leaved or herbaceous plants predominantly inhabiting (seasonally) dry environments, while large-leaved and woody taxa tend to be associated with tropical, more humid habitats (see Luebert et al., 2011a for an example from Neotropical *Heliotropium*).

Classification

The molecular data presented here resolve questions with regard to the phylogeny of Boraginales and allow a largely unequivocal subdivision into several clearly circumscribed families. In Boraginales I, the situation is straightforward, with three distinctive families recognized, as follows.

Codonaceae with a polymerous calyx, corolla and androecium and ovoid, many-seeded, capsular fruit with an apical style.

Wellstediaceae with a tetramerous calyx, corolla and androecium with an (at maturity) obcordate, two- to four-seeded capsule with immersed style insertion.

Boraginaceae with a pentamerous calyx, corolla, and androecium, the usual occurrence of faucal scales and a four-parted ovary, with a gynobasic style often surrounded by a ring nectary, maturing into four individual nutlets.

In Boraginales II, the situation is more complex. Analyses of ITS1 sequence data (Gottschling et al., 2001) and chloroplast sequence data (this study) recognize the same two major lineages of core Hydrophyllaceae, but they differ in whether the

group is monophyletic or not. It is therefore premature to decide whether the family should be split into two separate families, or left as a single family, perhaps with two subfamilies, but the groups can be circumscribed as follows:

Hydrophyllaceae I (Hydrophyllaceae s.str. = Hydrophyllaeae Rchb. and Romanzoffiaeae Dumort.): annual or perennial herbs; petal aestivation contorted or imbricate, corolla scales present, reduced or absent; style 1, ovary unilocular, with two large, fleshy parietal placentae filling the locule at anthesis or narrow cartilaginous placentae, ovary appearing two- or five-celled, nectary at ovary base reduced to glands or distinct disc; capsule dehiscing loculicidally with two valves.

Hydrophyllaceae II (Nameae Choisy): shrubs or small trees, rarely annual or perennial herbs or dwarf shrubs (only *Nama*); petal aestivation imbricate, corolla scales absent; styles 2, distinct to base or connate 3/4 of length, ovary bilocular, placentae narrow, membranaceous or cartilaginous, completely dividing ovary; capsule appearing two-celled, dehiscing loculicidally or loculicidally and septically, by two or four valves.

Heliotropiaceae: shrubs or trees or herbs, with a ring-shaped stigma overtopped by a sterile, conically elongated stigmatic column and fruits containing a multilayered, hard endocarp (usually completely divided into four-one-seeded or two-two-seeded parts).

Ehretiaceae: usually shrubs or trees, with the combination of flowers exhibiting an apical or rarely subapical forked style with two stigmas, fruits with a multilayered endocarp usually dividing into four-one-seeded or, less frequently, two-one-seeded pyrenes, rarely entire. An exclusive relationship of Lennoaceae with core-Ehretiaceae is strongly supported in this study, but they are morphologically anomalous parasites, with greatly reduced vegetative organs and a multiplication of floral parts, and having two ovules per locule.

Cordiaceae: usually trees or shrubs, with trait combination of apical styles that are twice divided with four stigmas, usually single-seeded fruits, with an entire multilayered endocarp, and seedlings with plicate cotyledons. *Coldenia* and *Hoplestigma* are morphologically distinct from the Cordiaceae as here defined. *Coldenia* is herbaceous and has four separate nutlets at maturity. *Hoplestigma* has an only once forked style and an increased stamen number (20–30).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. List of taxa, voucher information and GenBank accessions included in the phylogenetic study of Boraginales.